

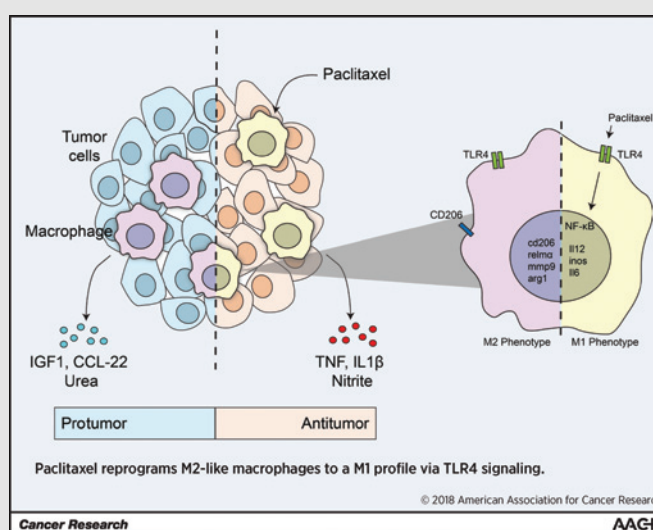
Paclitaxel Reduces Tumor Growth by Reprogramming Tumor-Associated Macrophages to an M1 Profile in a TLR4-Dependent Manner



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Abstract

Paclitaxel is an antineoplastic agent widely used to treat several solid tumor types. The primary mechanism of action of paclitaxel is based on microtubule stabilization inducing cell-cycle arrest. Here, we use several tumor models to show that paclitaxel not only induces tumor cell-cycle arrest, but also promotes antitumor immunity. *In vitro*, paclitaxel reprogrammed M2-polarized macrophages to the M1-like phenotype in a TLR4-dependent manner, similarly to LPS. Paclitaxel also modulated the tumor-associated macrophage (TAM) profile in mouse models of breast and melanoma tumors; gene expression analysis showed that paclitaxel altered the M2-like signature of TAMs toward an M1-like profile. In mice selectively lacking TLR4 on myeloid cells, for example, macrophages (LysM-Cre^{+/-}/TLR4^{fl/fl}), the antitumor effect of paclitaxel was attenuated. Gene expression analysis of tumor samples from patients with ovarian cancer before and after treatment with paclitaxel detected an enrichment of genes linked to the M1 macrophage activation profile (IFN γ -stimulated macrophages). These findings indicate that paclitaxel skews TAMs toward an immunocompetent profile via TLR4, which might contribute to the antitumor effect of paclitaxel and provide a rationale for new combination regimens comprising paclitaxel and immunotherapies as an anticancer treatment.



Significance: This study provides new evidence that the antitumor effect of paclitaxel occurs in part via reactivation of the immune response against cancer, guiding tumor-associated macrophages toward the M1-like antitumor phenotype.

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Introduction

Recent advances in understanding the mechanisms of action of classical anticancer drugs have unraveled the notion that in addition to antiproliferative effects, they might also interfere in the immune response in the tumor microenvironment (1). For instance, low doses of cyclophosphamide have induced T regulatory cell (Treg) inhibition (2). Anthracycline-based neoadjuvant chemotherapy increases the CD8⁺/Treg ratio (3), and the inhibitors of cyclin-dependent kinase 4/6 (CDK4/6) increase the tumor immunogenicity and reduce Treg populations in the tumor milieu (4). In line with this view, previous reports indicate that the antimicrotubule drug paclitaxel shows an alternative lipopolysaccharide (LPS)-like immunostimulatory effect in rodent and human cells (5–8). However, whether this paclitaxel activity could modulate the antitumor immune response and account for the therapeutic efficacy of paclitaxel is still unknown.

Macrophages are immune cells able to adopt different functional phenotypes in response to cytokine or pathogenic signals (10). The two major phenotypes, M1- and M2-, represent two points in a wide-spectrum contrasting functions; where M1-macrophages have proinflammatory, immunogenic, and antitumor properties, whereas M2-macrophages show anti-inflammatory, tolerogenic, and protumor effects (9–11).

In the tumor microenvironment, local mediators can influence tumor-associated macrophages (TAM) to adopt a M2-like phenotype, which supports tumor immunosurveillance escape, angiogenesis, and matrix remodeling contributing to tumor progression (11). Increased TAM infiltration in tumor tissue is related to poorer outcomes in the clinical setting (12). Therefore, the idea of exploring the macrophage plasticity through reprogramming TAM phenotype to display an M1-active phenotype is emerging as a potential therapeutic approach for cancer management (13–15).

Functionally M2-polarized macrophages by Th2 cytokines (IL4 and IL13) could be reprogrammed to M1 profile when exposed to LPS or other innate stimulus (9, 16, 17). Previous reports demonstrated in *in vitro* and *in vivo* conditions that paclitaxel causes LPS-like activation of the TLR4 receptor to induce proinflammatory response (6, 18–20). However, evidences about the paclitaxel/TLR4 interaction on macrophages plasticity and its impact on paclitaxel antitumor efficacy are still unknown. In the current study, we demonstrated that paclitaxel reprograms TAMs into a proinflammatory M1 profile via TLR4 signaling, which contributed, at least in part, to the antitumor effect of paclitaxel.

Materials and Methods

Animals

Mice C57BL/6 wild type (WT), knockout for Toll-like receptor 4 (TLR4^{-/-}) and Toll-like receptor-2 (TLR2^{-/-}) and BALB/c mice (WT) were obtained from the animal facility of the Ribeirão Preto Medical School, University of São Paulo (São Paulo, Brazil) used at the age of 6 to 8 weeks. Cre-recombinase homozygous mice under control of the myeloid-specific lysozyme M promoter (LysM-Cre^{+/+}) and TLR4-floxed mice (TLR4^{fl/fl}) were purchased from The Jackson Laboratory and bred in our facility. The macrophage-specific TLR4-deficient mice were generated initially by crossing LysM-Cre^{+/+} and TLR4^{fl/fl} mice. Following that, mice with LysM-Cre^{+/-}/TLR4^{fl/fl} genotype from F2 generation were interbred with TLR4-floxed mice (TLR4^{fl/fl}) to create the desired conditional mice with LysM-Cre^{+/-}/TLR4^{fl/fl} genotype. The back-

ground control LysM-Cre^{+/-} mice were produced crossing LysM-Cre^{+/+} with WT mice. Genotype of conditional mice was confirmed by standard PCR analysis. Primer pairs: LysM-Cre - mutant (5'-CCCAGAAATGCCAGATTACG-3'), common (5'-CTTGGC-TGCCAGAATTTCTG-3') and WT (5'-TTACAGTCGGCCAGGCTG-AC-3'); TLR^{fl/fl} - forward (5'-TGACCACCCATATTGCCTATAC-3') and reverse - (5'-TGATGGTGTGAGCAGGAGAG-3'). To confirm the TLR4 deletion, bone marrow-derived macrophages (BMDM) from LysM-Cre^{+/-} and LysM-Cre^{+/-}/TLR4^{fl/fl} mice were incubated with TLR4 ligand (LPS) or paclitaxel (30 μmol/L) for 24 hours. The BMDMs from conditional mice exhibited reduced levels of TNFα and IL6 in the supernatant compared with control. All animals were kept under controlled environmental conditions (12 hours of light/dark cycle, 55% ± 5% humidity, 23°C) with food and water *ad libitum*. The institutional Ethics Committee in Animal Experimentation (CEUA) of the Ribeirão Preto Medical School, University of São Paulo (protocol number: 56/2016) approved the experimental protocols.

BMDM polarization

Murine BMDMs were isolated and cultured in RPMI medium (Sigma) containing 10% FBS (v/v; Gibco), penicillin (100 U/mL), amphotericin B (2 μg/mL), and 20% of L929 cell culture supernatant (v/v) for 7 days, at 37°C in a 5% CO₂ atmosphere. After differentiation, the cells were seeded at a density of 1 × 10⁶ cells per well in 12-well plates and stimulated with medium (negative control - M0), LPS (100 ng/mL, M1-like - Sigma-Aldrich), paclitaxel (10, 30, 100 μmol/L, Sigma-Aldrich), IL4 (10 ng/mL, M2-like, R&D Systems), or IL4 plus paclitaxel or LPS; after 48 hours, M1 and M2 markers were analyzed (TNFα, IL12p40, IL6, nitrite, CCL22, IGF1, CD206, and urea, respectively).

NF-κB reporter assay

The murine macrophage cell line RAW 264.7, stably expressing luciferase under control of NF-κB responsive promoter (pNF-κB-Luc), was cultured in RPMI medium (Sigma) containing 10% FBS (v/v), penicillin (100 U/mL), and amphotericin B (2 μg/mL), at 37°C in a 5% CO₂ atmosphere. The RAW 264.7 cells (3 × 10⁵ cells/well) were cultured overnight in 24-well plates. Cells were incubated with LPS (100 ng/mL) or paclitaxel (10, 30, 100 μmol/L). Unstimulated macrophages acted as a negative control. Intracellular contents were extracted in lysis buffer (TNT), and the luciferase activity was determined on a luminometer (FlexStation 3 Microplate Reader) using the dual luciferase reporter assay system (Promega) according to the manufacturer's directions. Results are expressed as relative luciferase activity (fold difference compared with negative control).

Cytokine and chemokine levels

The levels of cytokines and chemokines present in the supernatants from BMDM cell culture were quantified by ELISA using antibodies obtained from R&D Systems according to the manufacturer's instructions. The optical density of the individual samples was measured at 450 nm (Spectra Max-250, Molecular Devices) as described previously.

In vivo tumor models

Breast cancer 4T1 (ATCC) or melanoma B16 (ATCC) cells lines were cultured in RPMI containing 10% FBS (v/v), penicillin (100 U/mL) and amphotericin B (2 μg/mL). Prior to use, cells with 70% to 80% of confluence were detached with trypsin-EDTA 0.25% and washed in PBS twice. For breast cancer model, BALB/c

mice were subcutaneously inoculated with 5×10^4 cells of 4T1. After 16 days, the animals were randomly divided into two groups and treated with saline or paclitaxel (10 mg/kg, i.p., every 8 hours). The animals were euthanized 24 hours after the beginning of the treatment, and tumors were harvested to evaluate TAM phenotype. From melanoma models, C57BL/6, WT, TLR4^{-/-}, LysM-Cre^{+/-}, or LysM-Cre^{+/-}/TLR4^{fl/fl} mice were subcutaneously inoculated with 5×10^4 cells of B16. After 7 days, the animals were divided in two groups treated with saline or paclitaxel (10 mg/kg, i.p.). Tumor volumes were calculated according to the formula: tumor volume (mm³) = $L \times W^2/2$, where L represents the major axis (largest cross-sectional diameter) of the tumor, and W represents the minor axis.

TAM isolation

Total tumor was minced and incubated with collagenase type II (1 mg/mL, Sigma) for 40 minutes at 37°C. Then, cell suspension was carefully layered on Percoll (70%/30%) and centrifuged (1,800 RPM, 23 minutes, 4°C). The lymphomononuclear correspondent layer was isolated and cultured for 40 minutes at 37°C. After three washes, the adherent cells remaining in the plate were removed using a cell scraper (21). M1 and M2 markers were evaluated by flow cytometry (CD11b⁺F4/80⁺CD206⁺) and real-time PCR (M1-*Il12*, *Inos*, *Il6*, and M2- *cd206*, *Relm α* , *mmp9*, and *Arg1*).

Gene set enrichment analysis

All microarray data reported in this article are from Gene Expression Omnibus (GEO) database (accession no. GSE15622).

Gene set enrichment analysis (GSEA) was performed using the GSEA software from the Broad Institute (software.broadinstitute.org/gsea/index.jsp). The complete list of genes and their scores were used with a focus on immunologic signatures collection (*immuneSigDB*) composed of gene sets that represent cell types, states, and perturbations within the immune system (22). The FDR Q value represents estimated probability of false positive finding (FDR $q < 0.25$ was accepted), and the nominal P value estimates the statistical significance of the enrichment score for a single gene set.

Statistical analysis

The data are expressed as mean \pm SEM. All *in vitro* experiments were performed in three independent experiments. The means of different treatments were compared by Student t test or one- or two-way ANOVA, followed by Bonferroni test as appropriate. Statistical significance was accepted when $P < 0.05$. All of the statistical analyses were performed using GraphPad Prism version 7.0c (GraphPad Software).

Results

Paclitaxel promotes macrophage differentiation toward M1 phenotype through the TLR4 pathway

To investigate the potential of paclitaxel to polarize macrophages, BMDMs were cultured with LPS or paclitaxel. Similar to LPS, paclitaxel induced an increase in M1 markers (TNF α , IL12, and nitrite) in a concentration-dependent manner (Fig. 1A–C).

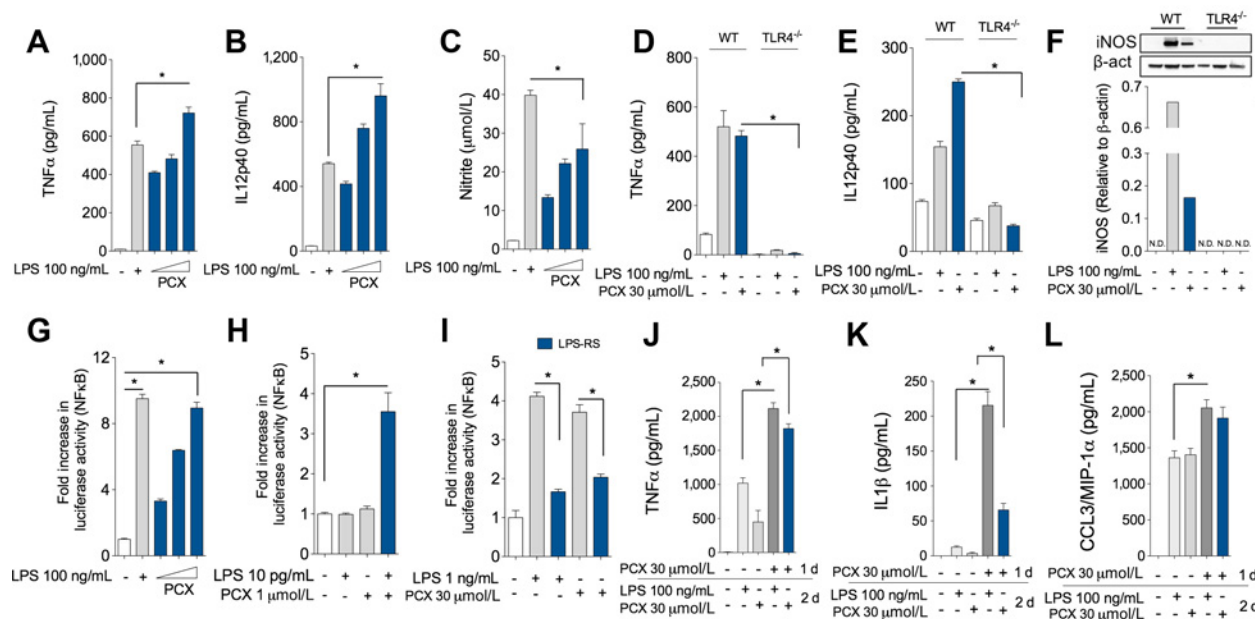


Figure 1.

Paclitaxel (PCX) induced M1 macrophage polarization via TLR4. BMDMs were stimulated with LPS (100 ng/mL) or paclitaxel (10, 30, and 100 μ mol/L) for 48 hours; then, supernatants were collected, and the following M1 markers were measured: TNF α (A), IL12p40 (B), and nitrite (C). BMDMs from WT or TLR4^{-/-} mice were stimulated with LPS (100 ng/mL) or paclitaxel (30 μ mol/L) for 48 hours and the following M1 markers were measured: TNF α (D), IL12p40 (E), and iNOS expression (F); unstimulated cells acted as a negative control (M0). G, RAW 264.7 cells transfected with pNF- κ B-Luc were stimulated for 6 hours with LPS (100 ng/mL) or paclitaxel (10, 30, 100 μ mol/L). H, The RAW 264.7 cells were stimulated with low doses of LPS (10 pg/mL), paclitaxel (1 μ mol/L), or LPS (10 pg/mL) plus paclitaxel (1 μ mol/L). I, The RAW 264.7 cells were preincubated with LPS-RS (TLR-4 antagonist; 500 ng/mL for 30 minutes) and then stimulated with LPS (1 ng/mL) or paclitaxel (30 μ mol/L); unstimulated RAW 264.7 cells acted as a negative control. The results are expressed as relative luciferase activity (fold difference compared with negative control). BMDMs were stimulated with paclitaxel for 24 hours and cells were then washed and restimulated with LPS or paclitaxel for 24 hours; unstimulated BMDMs acted as a negative control (M0). The supernatants were collected, and the following proinflammatory cytokines were measured: TNF α (J), IL1 β (K), and CCL3/MIP-1 α (L). All values are means \pm SEM ($n = 3$); *, $P < 0.05$.

Paclitaxel-induced M1 polarization was abrogated in BMDMs from TLR4^{-/-} mice (Fig. 1D–F), but not in BMDMs from TLR2^{-/-} mice (Supplementary Fig. S1A–S1C). Paclitaxel was also able to induce NF-κB signaling in RAW-luc macrophages (Fig. 1G), which correlates with previous established reports that TLR4/NF-κB signaling guides macrophages toward an M1 phenotype (10). Paclitaxel associated with LPS, at low doses, also promoted NF-κB activation (Fig. 1H). Furthermore, the LPS-RS (TLR4 antagonist) blocked the NF-κB activation induced by paclitaxel (Fig. 1I). Paclitaxel, at the indicated dose, did not induce macrophage apoptosis (Annexin V–stained cells ≤10%) or reduce the relative amounts of viable cells (MTT test; Supplementary Fig. S2A–S2E). Paclitaxel-primed macrophages responded to a second exposure to LPS or paclitaxel producing higher amounts of inflammatory cytokines (TNFα, IL1β, and CCL3/MIP-1α; Fig. 1J–L). These results indicated that paclitaxel drives macrophages to an M1 profile via TLR4.

Paclitaxel impairs M2 macrophage polarization in a TLR4-dependent manner

Given that paclitaxel effectively drove BMDMs to an M1 profile, we assessed whether paclitaxel could impair M2 polarization. For this, we compared the responses of IL4 M2-polarized macrophages with the responses of BMDMs concomitantly cultured with IL4 plus paclitaxel. We found that paclitaxel treatment reduced the levels of M2-specific markers (CD206, CCL-22, IGF1, and urea; Supplementary Fig. S3A–S3G). Moreover, paclitaxel boosted M1 markers (TNFα, IL6, and nitrite) even in the presence of IL4 similarly to positive control LPS (Supplementary Fig. S3H–S3J). Notably, it was observed that M1-triggered cells with paclitaxel displayed similar production of inflammatory cytokines compared with cells treated with IL4 plus paclitaxel concomitantly (Supplementary Fig. S3H–S3J). Furthermore, the changes induced by paclitaxel toward M1 on IL4-stimulated macrophages were dependent on TLR4. In agreement, TLR4-

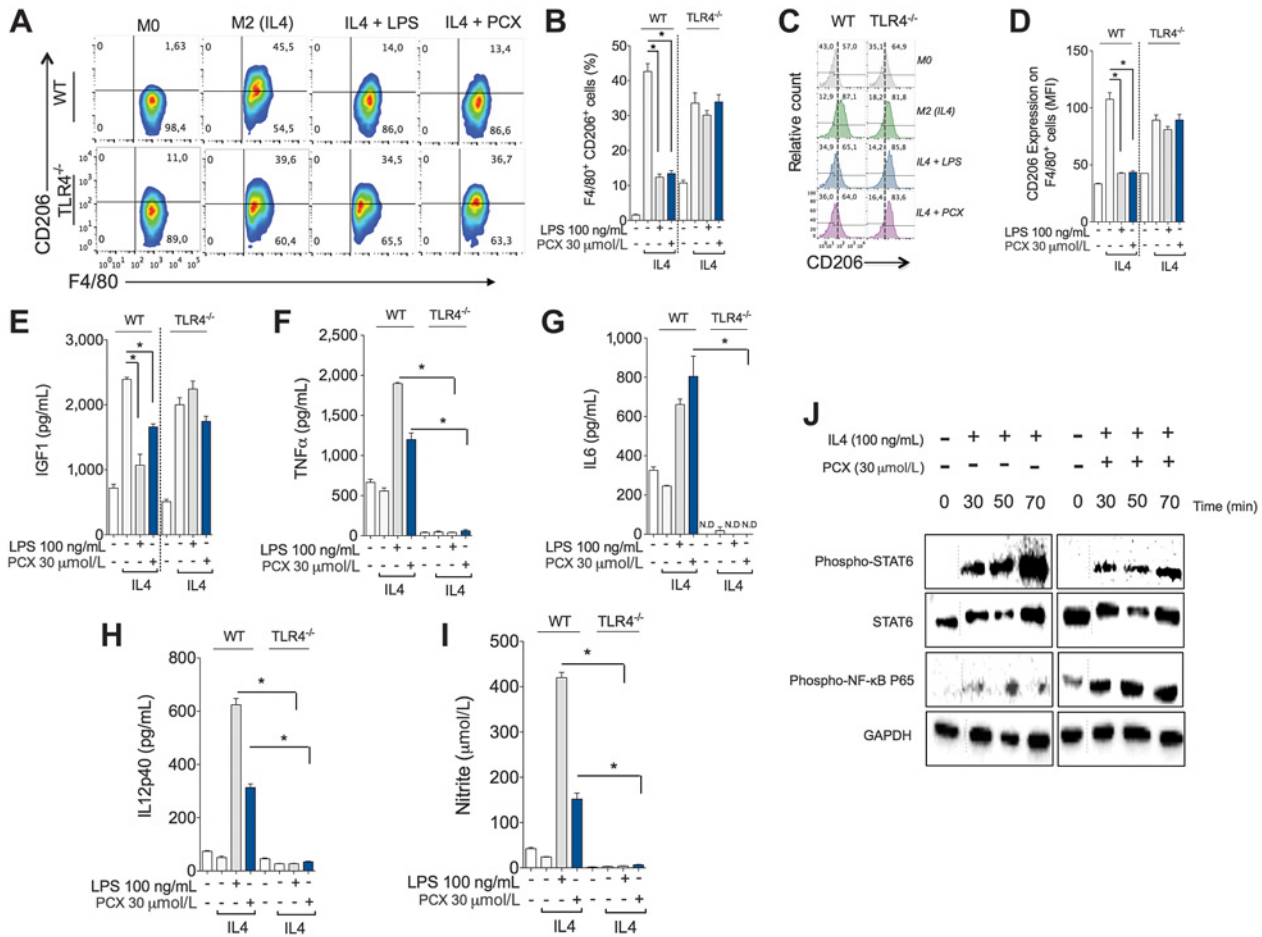


Figure 2.

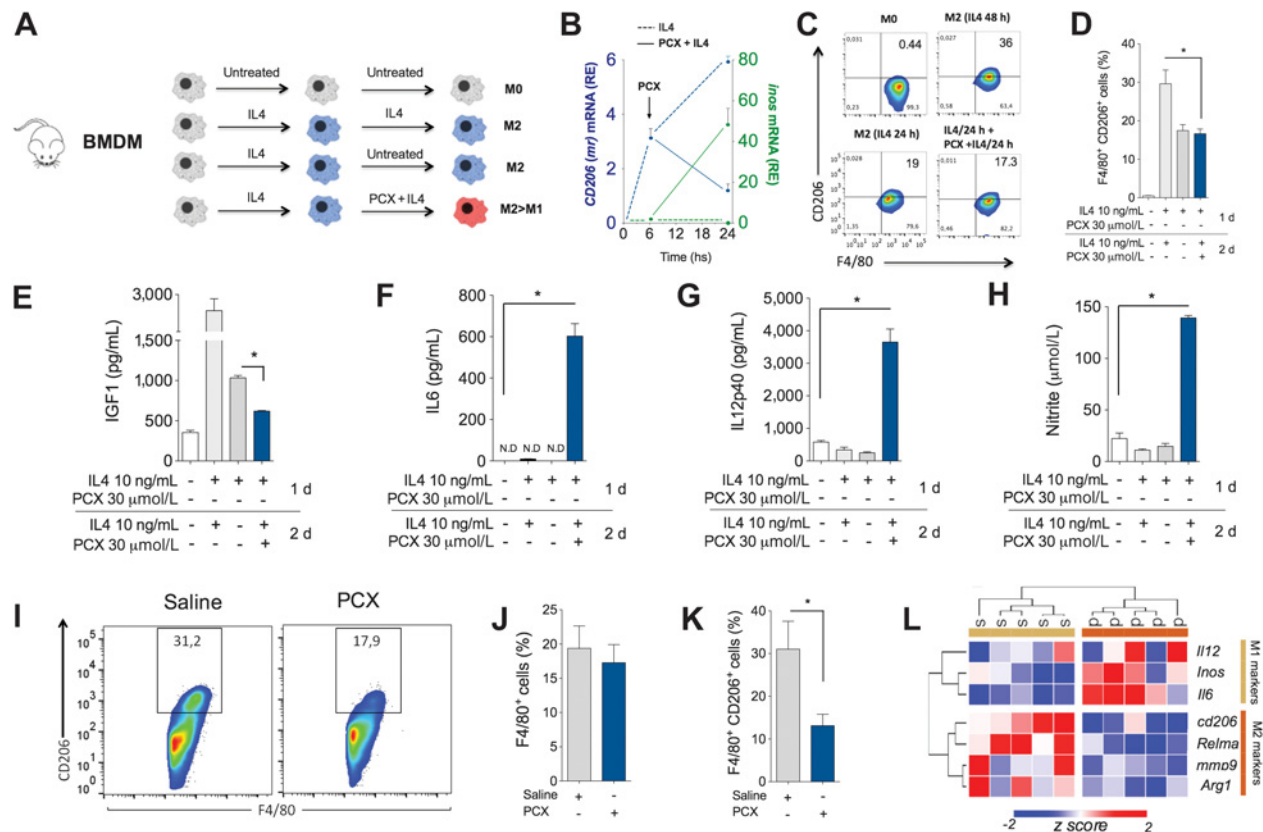
Paclitaxel (PCX) impaired M2 macrophage polarization in a TLR4-dependent manner. BMDMs from WT or TLR4^{-/-} mice were cultured with IL4 (10 ng/mL) alone or concomitantly stimulated with IL4 plus LPS (100 ng/mL) or IL4 plus paclitaxel (30 μmol/L); unstimulated cells acted as a negative control (M0). **A**, Representative FACS plots of F4/80⁺CD206⁺ staining on BMDM cells. **B**, Quantification of the results from **A**. **C**, Representative histogram plots comparing the mean fluorescence intensity (MFI) of CD206 on F480⁺ cells, incubated with medium (gray), IL4 (green), IL4 plus LPS (blue), or IL4 plus paclitaxel (rose). **D**, Quantification of the results from **C**. Macrophage markers, such as cytokines and nitrite production, were measured in the supernatants: M2 marker, IGF1 and M1 markers (**E**), TNFα (**F**), IL6 (**G**), IL12p40 (**H**), and nitrite (**I**). **J**, BMDMs were cultured with IL4 (10 ng/mL) alone or stimulated with IL4 and after 10 minutes with paclitaxel (30 μmol/L); unstimulated cells acted as a negative control (M0). The samples were collected 0, 30, 50, and 70 minutes after stimulus, and the following markers were measured by Western blotting: phospho-STAT6, STAT6, and phospho-NF-κB P65. All values are means ± SEM (*n* = 3); *, *P* < 0.05.

deficient BMDMs treated with IL4 plus paclitaxel preserved the expression of M2 markers (CD206 and IGF1; Fig. 2A–E), whereas the M1 markers (TNF α , IL6, IL12, and nitrite) were not increased (Fig. 2F–I). STAT6 pathway is critical for IL4-induced M2 profile, while NF- κ B is essential for TLR agonist-associated M1 polarization (10). In view of the paclitaxel effect on TLR4 receptors, we investigated the dynamic changes of these transcription factors during IL4 and paclitaxel concomitant stimuli. First, we observed that IL4 induced STAT6 phosphorylation in a time-dependent manner. However, the treatment with paclitaxel reduced IL4-induced STAT6 phosphorylation. On the other hand, phospho-P65 (a NF- κ B subunit) levels were enhanced by paclitaxel (Fig. 2J). Collectively, these findings indicate that paclitaxel impaired IL4/STAT6-dependent M2 polarization by a TLR4/NF- κ B pathway.

Paclitaxel reprograms M2 macrophages toward an M1 antitumor profile

Previous reports have demonstrated that LPS can reprogram M2 macrophages to an M1 profile (9, 16, 17). We assessed

whether paclitaxel could repolarize M2 cells to an M1-active phenotype. BMDMs were polarized to an M2 profile with IL4 and then stimulated with paclitaxel (Fig. 3A). After 6 hours of IL4 stimulus, an increase in *cd206* expression was detected (M2 marker), which was maintained for 24 hours. No effect was observed in *inos* (M1 marker) expression (dashed line, Fig. 3B). However, when paclitaxel was added to M2-polarized cells, the expression of *cd206* was downregulated whereas the inflammatory M1 marker *inos* was upregulated (compact line, Fig. 3B). Next, we studied the response of paclitaxel-treated M2 cells. For this, BMDMs were completely differentiated to an M2 phenotype under IL4 stimulus for 24 hours. Then, cells were washed and restimulated with IL4 alone (as a control), or IL4 plus paclitaxel. We found that M2-polarized macrophages subsequently stimulated with paclitaxel displayed reduced M2 markers (CD206 and IGF1) and showed a significant increase in proinflammatory cytokine secretion and iNOS-mediated nitric oxide (NO) production (Fig. 3C–H). To determine whether our findings were reproducible in *in vivo* conditions, mice bearing breast tumors were treated with paclitaxel or vehicle and the M1 and M2 populations



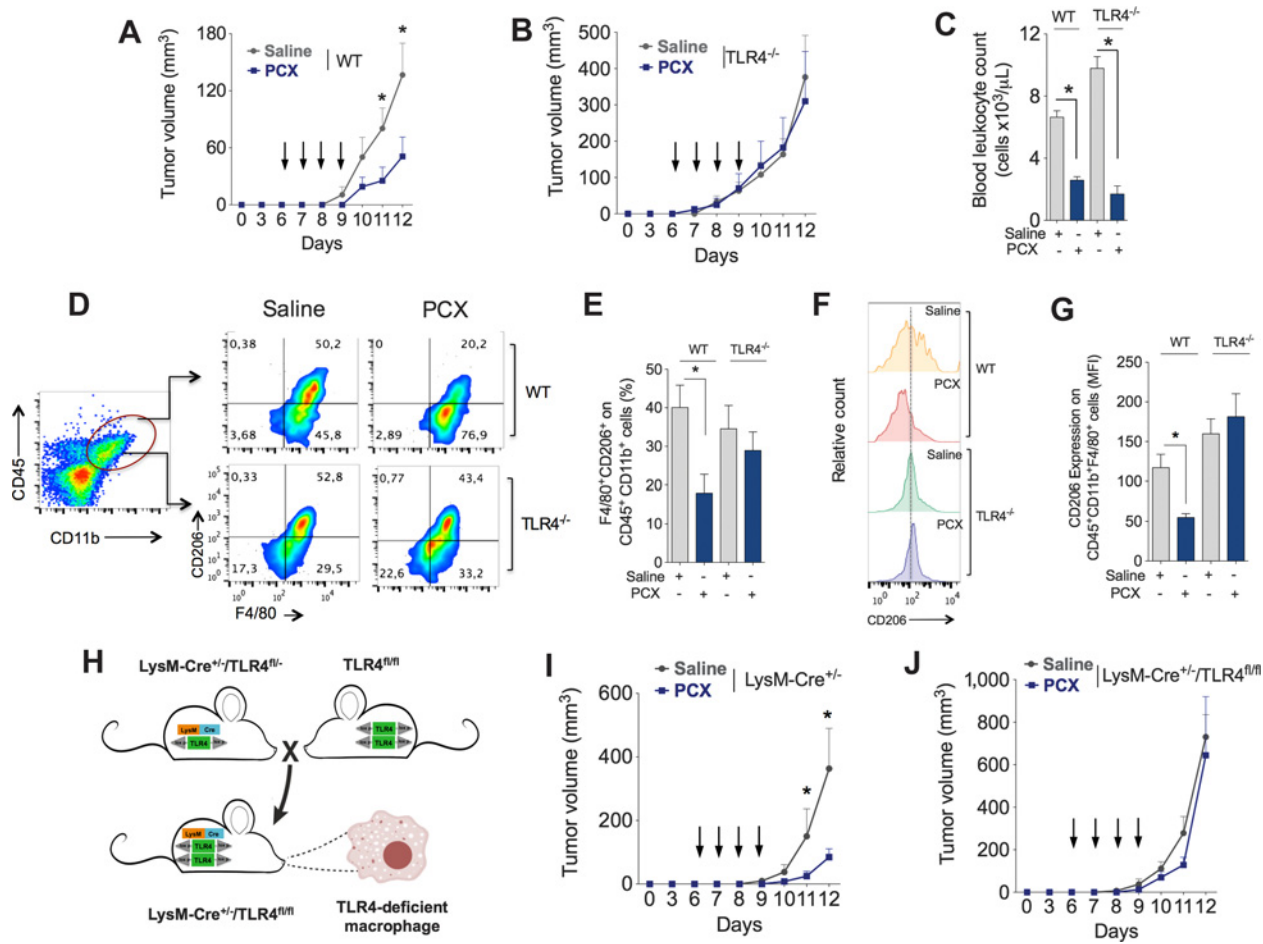


Figure 4.

Paclitaxel (PCX) reduced tumor growth by shifting TAMs to a less immunotolerant profile via TLR4. Melanoma (B16)-bearing mice were treated with saline or paclitaxel. **A** and **B**, Melanoma (B16) growth curves in WT and TLR4^{-/-} animals. Arrows, days on which paclitaxel (10 mg/kg, i.p.) or saline were administered. **C**, Blood leukocyte cell count in WT and TLR4^{-/-} control and paclitaxel-treated mice. Whole tumors were processed and cells F4/80⁺ on CD45⁺/CD11b⁺ were quantified by flow cytometry. **D**, Representative FACS plots of F4/80⁺/CD206⁺ on CD45⁺/CD11b⁺. **E**, Quantification of the results from **D**. **F**, Representative histogram plots comparing mean fluorescence intensity (MFI) of CD206 on CD45⁺CD11b⁺F480⁺ cells. **G**, Quantification of the results from **F**. **H**, Breeding scheme for conditional mice. The LysM-Cre^{+/+}/TLR4^{fl/fl} mice from F2 generation were interbred with TLR4-floxed mice (TLR4^{fl/fl}) to create the desired conditional mice (LysM-Cre^{+/+}/TLR4^{fl/fl}). The control LysM-Cre^{+/+} mice were produced crossing LysM-Cre^{+/+} with C57BL/6 WT mice. **I** and **J**, Melanoma (B16) growth curves in LysM-Cre^{+/+}/TLR4^{fl/fl} mice. Arrows, days on which saline or paclitaxel (10 mg/kg, i.p.) was administered. All values are means ± SEM; *, P < 0.05.

were analyzed. Although the frequency of TAMs (F4/80⁺) was not affected, the amounts of M2 macrophages (F4/80⁺/CD206⁺) were reduced in paclitaxel-treated mice (Fig. 3I-K). Furthermore, the gene expression analysis in TAMs isolated from tumor revealed that paclitaxel treatment altered the signature from an M2-like profile (*cd206*, *relmα*, *mmp9*, and *arg1*) to an M1-like antitumor profile (*Il12*, *inos*, and *Il6*; Fig. 3L). Thus, paclitaxel effectively repolarized M2 macrophages to a proinflammatory state in *in vitro* and *in vivo* conditions.

Paclitaxel induces tumor regression by shifting TAMs to an M1 profile via TLR4

Next, we sought to determine whether the paclitaxel effect driving TAMs to the active M1 phenotype could have therapeutic potential. Cutaneous melanoma is a highly aggressive tumor that often shows resistance to antiproliferative chemotherapy (23).

However, melanoma seems more sensitive to immunotherapy than other neoplasms (23). Therefore, to evaluate the immunomodulatory effect of paclitaxel, murine melanoma cell line (B16) was injected into WT and TLR4 knockout mice, which were treated with paclitaxel or vehicle. Whereas paclitaxel treatment reduced tumor growth in the WT animals, it was less effective in TLR4-deficient mice (Fig. 4A and B). Importantly, the antiproliferative action of paclitaxel seems to be similar in WT compared with TLR4-deficient mice, because reduction in the blood leukocyte count after paclitaxel treatment was not different in these two mouse strains (Fig. 4C). In accordance with our previous results, we found that melanoma-bearing mice treated with paclitaxel showed reduced TAMs positive to CD206 (M2 marker). However, this effect was not observed in TLR4-deficient mice (Fig. 4D-G), suggesting that the immune mechanism of action of paclitaxel involves TAM polarization via TLR4 activation. TAMs are mostly

derived from Ly6C⁺ circulating monocytic precursors differentiated into macrophages in the tumor tissue (24). Therefore, we investigated the effects of paclitaxel treatment in the phenotype of tumor mature/immature macrophage subsets. First, we observed that paclitaxel treatment increased the percentage of tumor-infiltrating myeloid cells (CD45⁺CD11b; Supplementary Fig. S4A and S4B). The myeloid infiltrate was primarily comprised of mature TAMs (MHCII⁺F4/80⁺Ly6C⁻), with a lesser extent of immature macrophages (MHCII⁺F4/80⁺Ly6C⁺) and monocytes (F4/80⁻Ly6C⁺; Supplementary Fig. S4C–S4F). Although paclitaxel treatment increased the infiltration of monocytes (MHCII⁻F4/80⁻Ly6C⁺) at the time of evaluation, these cells did not express differentiation markers, such as MHCII or F4/80 (Supplementary Fig. S4G). Next, to characterize the polarization status of monocytes/macrophages, we analyzed the expression of CD206, an M2 marker. The large fraction of myeloid cells (CD45⁺CD11b⁺) that expressed CD206 are mature TAMs (CD11b⁺F4/80⁺Ly6C⁻; Supplementary Fig. S4H–S4J). Strikingly, paclitaxel treatment reduced CD206 and increased MHCII expression on mature TAMs (CD11b⁺F4/80⁺Ly6C⁻) compared with control (Supplementary Fig. S4K). These results suggest that the paclitaxel effect is mainly on mature M2-polarized TAMs. To confirm this hypothesis, we generated a conditional mice in which TLR4 was specifically deleted from myeloid cells (e.g., macrophages; *LysM-Cre^{+/-}/TLR4^{fl/fl}*) and the *LysM-Cre^{+/-}* mice were used as background control (Fig. 4H; Supplementary Fig. S5A–S5C). The antitumor effect of paclitaxel in *LysM-Cre^{+/-}/TLR4^{fl/fl}*

mice was significantly reduced compared with control (Fig. 4I–J). Altogether, these results suggest that paclitaxel elicits an immune response in the tumor microenvironment via TLR4 signaling and directly drives mature TAMs to a less immunotolerant profile contributing to the antitumor actions of paclitaxel.

Paclitaxel treatment alters immunologic gene signature in the tumor microenvironment

We also tested whether our preclinical data could be extended to patients. For this, we used the following criteria: (i) obtained data from patients with cancer treated with paclitaxel monotherapy; and (ii) obtained data from tumors sensitive to immunotherapy. Ovarian cancer is a malignancy in which paclitaxel may be used as a first-line treatment (25). Furthermore, new evidence suggests that ovarian cancers are potentially sensitive to immunotherapy (26). Therefore, we used a GEO dataset (GSE15622) microarray gene file from patients with ovarian cancer that had a biopsy performed before and after 3 cycles of paclitaxel monotherapy. GSEA with a focus on immunologic signatures collection (*immuneSigDB*; ref. 22) showed that paclitaxel treatment downregulated genes within GSEA terms linked to "memory T CD4⁺ cells," "dendritic cells CD11b⁺," and "CD4⁺ FOXP3⁺ cells" (Fig. 5A). Strikingly, a highly significant enrichment of the M1-like macrophage genes sets "INFG stimulated macrophage" and "INFG plus PAM3Cys stimulated macrophage" was found upregulated in the paclitaxel-treated

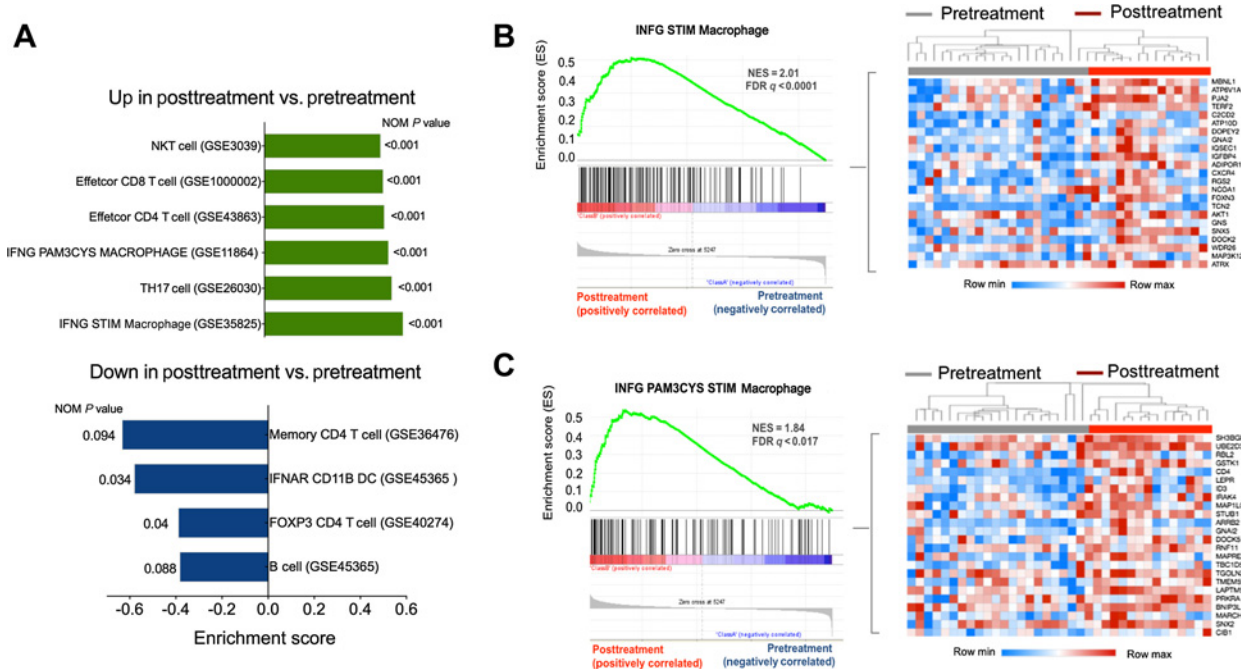


Figure 5. GSEA of the paclitaxel response in patients with ovarian cancer. Gene expression profile from human ovarian cancer biopsies obtained before and after three cycles of paclitaxel chemotherapy (GSE15622) was explored by GSEA. **A**, Representative GO terms enriched for up- and downregulated genes posttreatment versus pretreatment with paclitaxel; the enrichment score (ES) is indicated in the x-axis. **B** and **C**, The genes were evaluated by microarray data and compared with the gene set available from GEO under accession no. GSE35825 -UNTREATED_VS_IFNG_STIM_MACROPHAGE_DN (**B**) and GSE11064-UNTREATED_VS_IFNG_PAM3CYS_STIM_MAC_UP (**C**). The green curve shows the running enrichment score for the gene set as the analysis walks down the ranked gene list; the leading edge for the gene set is shown as short bars at the bottom. Expression heatmaps of the corresponding 23 most significant genes ranked based on the signal-to-noise ratio. Dark red, higher expression; blue, lower expression.

patients with reported FDR Q values of <0.0001 and 0.017 , respectively (Fig. 5B and C). These signatures showed similar trends observed in the tumors from paclitaxel-treated mice and demonstrate a potential clinical relevance of our experimental findings.

Discussion

The antiproliferative effects of paclitaxel have long been explored in medical oncology to treat a variety of malignant neoplasms. Our study provides new evidence that the antitumor effect of paclitaxel, at least in part, occurs through the modulation of the immune response. Along this line, paclitaxel reprogrammed immunotolerant M2-polarized macrophages into an M1-like phenotype via TLR4 activation. This effect was reproduced *in vivo* in the tumor microenvironment and was related to antitumor outcomes. Furthermore, clinical tumor samples from paclitaxel-treated patients showed an enrichment of genes linked to an M1-like macrophage profile.

An emerging concept suggests that the therapeutic efficacy of several classical anticancer drugs also relies on their capacity to modify the immune response in the tumor milieu (1–4). In this regard, paclitaxel is an antiproliferative drug that has been assigned immunostimulatory properties (5–8). However, how paclitaxel induces immunomodulation and how this mechanism could change the immune response against cancer in the tumor microenvironment is unknown. Here, we identified that paclitaxel induced M1 macrophage polarization via TLR4/NF- κ B signaling. Macrophages exposed to paclitaxel exhibited an increase in M1 markers (TNF α , IL12, and iNOS), and this effect was not observed in cells lacking TLR4. Furthermore, the exposure to paclitaxel led to an increase in the NF- κ B activity, which was inhibited by a TLR4 antagonist. The immunostimulatory effect of paclitaxel mediated by TLR4 was demonstrated in other antigen-presenting cells (APC). In accordance, Pfannenstiel and colleagues (27) demonstrated that paclitaxel treatment increased early dendritic cell maturation and function. Paclitaxel via TLR4 activation enhanced the expression and production of costimulatory molecule IL12 and the ability of dendritic cells to induce CD8⁺ T-cell responses. These observations offer support for the immune-related stimulatory effect of paclitaxel. Moreover, they could also suggest that the modulation of APC function could also be an additional mechanism by which paclitaxel reduces tumor burden.

Macrophage activation state is critical to balance between inflammation and resolution or tissue homeostasis and disease pathogenesis (28). This wide range of action is possible, because macrophages respond to environmental stimuli (cytokines, TLRs ligands, growth factors) adopting distinct activation states (9, 10). An extended version of the macrophage activation status recognizes M1/M2 as extremes of a functional spectrum composed by M1 and at least three subsets of M2: M2a (relative to exposure to IL4 or IL13), M2b (relative to exposure to immune complexes plus IL1 or LPS) and M2c (relative to exposure to IL10, TGF β , or glucocorticoids; refs. 28–30). The transcription factor STAT6 is critical for IL4-inducing M2 profile, whereas NF- κ B is involved in the TLR4-related M1 activation. In our study, paclitaxel treatment blocked the IL4/STAT6-dependent M2 polarization, while driving cells to M1 profile via NF- κ B activation. Similarly, the STAT6 activity was repressed in B-lymphocytes when concomitantly exposed to LPS and IL4 (31). This indicated that paclitaxel might

activate TLR4/NF- κ B to exert an antagonistic effect on IL4/STAT6-mediated macrophage polarization.

Contrasting with lymphocytes, where the activation leads to a Th1, Th2, or Th17 terminal fate, macrophages are plastic cells that are able to adjust their phenotype according the environmental signals (11). Previous studies have demonstrated that M2-polarized macrophages stimulated with LPS are effectively reprogrammed to the M1 phenotype (9, 16, 17). Therefore, we assessed whether paclitaxel could reprogram M2-polarized cells to M1 profile. Strikingly, after paclitaxel exposure, M2 macrophages clearly adopted an M1-like state, an effect that was not reproduced in TLR4-deficient cells. These results suggest that paclitaxel, similarly to LPS, could reprogram macrophages driving M2 cells to an M1 profile via TLR4 signaling.

In the tumor microenvironment, the TAMs adopt an immunosuppressive M2-like phenotype important to support cancer growth (10, 11). Therefore, we decided to investigate the effect of paclitaxel on TAM function. We observed that the TAMs isolated from tumor-bearing mice treated with paclitaxel showed a less immunotolerant profile associated with an antitumor effect, which did not occur in TLR4-deficient mice or in conditional mice with TLR4 deletion in myeloid cells (e.g., macrophages). Several studies have demonstrated that TAMs can influence response to chemotherapy (32–34). Mantovani and colleagues (34) showed that macrophage-inactivating agents reduce the efficacy of doxorubicin treatment in mice transplanted with leukemia and lymphoma. More recently, it was demonstrated that the activation of macrophages by treatment with class IIa histone deacetylase inhibitor improves the efficacy of chemotherapy and checkpoint blockade in mice with breast tumors (35). These findings suggest that the macrophage polarization status is important to effective control of tumor progression. However, pleiotropic and sometimes opposing effects may occur in the tumor microenvironment. For instance, although IL6 is produced by M1-active macrophages, its expression has been associated with poor clinical outcomes in patients with cancer (36). In the current study, we did not investigate the role of IL6 in the antitumor effect of paclitaxel, and its production was used only as a marker of differentiation between M1 and M2 macrophages. However, considering that M1-polarized TAMs produce a significant antitumor response (13–15), we believe that in our experimental conditions, the M1-derived IL6 is not exerting a relevant protumorigenic effect.

It is well known that TLR receptors play a critical role on the orchestration of the immune response in tumor microenvironment (37–39). In line with this view, we observed that the full TLR4 knockout mice and the conditional LysM-Cre^{+/–}/TLR4^{fl/fl} mice showed similarly increased tumor growth rates. Notably, this was a demonstration that the TLR4 activation on macrophages is important to control the tumor growth. These data are supported by the notion that the innate function of TLR4 in recognizing "danger signals," from bacterial components [pathogen-associated molecular patterns (PAMP)] or endogenous ligands from cell injury and/or death [damage-associated molecular patterns (DAMP)], is important to mount the host antitumor immune response (37–40). Accordingly, Coley demonstrated in the late 19th century that the injection of a mixture of killed pathogenic bacteria could treat patients with inoperable sarcomas (41). LPS was further isolated and described as the active agent of Coley's toxin (42). Later on, several reports showed that LPS, used as a single agent or in combination with other therapies,

can induce antitumor immunity in mice and humans (37, 43, 44). Goto and colleagues (44) showed that the treatment of patients with cancer with intradermal injection of LPS and cyclophosphamide induced a continuous release of cytokines (TNF α and IL6) and caused an antitumor response with less toxicity. Strikingly, patients with breast cancer treated with paclitaxel showed an increase in plasma levels of IL6 and IL8, indicating a proinflammatory effect of paclitaxel in humans (45). In accordance, our analysis demonstrated that patients treated with paclitaxel had an enrichment of genes linked to the inflammatory M1-like macrophage phenotype in the tumor microenvironment. These observations suggest that paclitaxel, like LPS, may produce an antitumor effect through immune response stimulation.

Although our *in vitro* evidences indicate a direct action of paclitaxel on the TLR4 receptor, *in vivo*, we cannot exclude a possible indirect involvement of DAMPs and PAMPs in the TLR4 activation. In response to chemotherapy treatment, malignant cells can dispatch DAMPs, such as high mobility group box 1 (HMGB1) that may interact with TLR4 and exert a potent adjuvant effect (1, 39). Several evidences indicate that PAMPs from microbiota can shape anticancer immune response and modulate the efficacy of antineoplastic drugs (46, 47). For instance, treatment with cyclophosphamide induced breaches in the intestinal epithelium and bacterial translocation to secondary lymphoid organs, resulting in the generation of antitumor immune response mediated by Th17 lymphocyte subset (46). The paclitaxel antiproliferative effect is also associated with gastrointestinal toxicity (48). Therefore, we could not discard the possibility that microbiota components may contribute for the immunostimulating effects of paclitaxel.

It is worth noting that the TLR4 activation by paclitaxel may be a double-edged sword, because this signaling seems to also be involved in paclitaxel side effects including the neuropathic pain development (8). Furthermore, it was demonstrated that the exposition of human macrophages and other cells to paclitaxel led to an increase in inflammatory cytokines via TLR-4 (6, 8, 18–20), but others showed the opposite (49).

Collectively, our data support the notion that the antitumor effect of paclitaxel, at least in part, is linked to TAM reprogramming to a less immunotolerant profile via TLR4 activation. There is a still unmet need to understand the potential immune effects of

paclitaxel in the clinical setting. Therefore, we suggest that converting M2-polarized TAMs toward a proinflammatory M1 phenotype with paclitaxel could be explored as a potential strategy to boost currently used immunotherapies in order to improve clinical outcomes for patients with cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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